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TCRα–CD3δε Association Is the Initial Step in αβ Dimer Formation in Murine T Cells and Is Limiting in Immature CD4⁺CD8⁺ Thymocytes

Kelly P. Kearse, Joseph L. Roberts, and Alfred Singer
Experimental Immunology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Summary

The present study has examined the molecular events leading to formation of αβ dimers in normal murine thymocytes and mature T cells. We demonstrate that TCR assembly proceeds by initial association of TCRα with CD3δε proteins and by association of TCRβ with CD3γε proteins to form αδε and βγε trimers; these trimers then associate to form αδε–βγε complexes, within which α–β disulfide bond formation occurs. We also show that TCR-associated protein (TRAP) associates uniquely with CD3γε pairs and that formation of βγε trimers occurs subsequent to TRAP dissociation. Importantly, we document that the assembly step that is quantitatively limiting in CD4⁺CD8⁺ thymocytes is the initial association of TCRα with CD3δε chains, which appears necessary to protect nascent TCRα proteins from accelerated degradation within the ER of immature thymocytes.

Introduction

The T cell antigen receptor (TCR) is a multisubunit complex composed of at least six different proteins from three distinct protein families: clonotypic α and β proteins, which are expressed as disulfide-linked heterodimers and determine the recognition specificity of the complex; invariant CD3 chains, which consist of noncovalently associated pairs of CD3γε and CD3δε proteins; and ζ family proteins, which exist either as disulfide-linked ζ–ζ homodimers or ζ–x heterodimers (Baniyash et al., 1988). The intracellular transport and expression of TCR proteins is a direct function of their assembly status. Unassembled CD3 and TCRαβ proteins and partial complexes of CD3 components are retained within the endoplasmic reticulum (ER), and, depending upon the particular chain, degraded (Chen et al., 1988; Lippincott-Schwartz et al., 1988). Intermediate TCR αβγδε TCR complexes lacking ζ exit the ER and transit through the Golgi system; however, they are targeted to lysosomes for degradation. Only complete TCR complexes containing all three families of TCR proteins are efficiently transported to the plasma membrane (Minami et al., 1987; Sussman et al., 1988; Wileman et al., 1990; Hall et al., 1991).

While much is known about the intracellular fate of individual TCR proteins, the molecular interactions important in the initial assembly of TCR complexes within the ER remain poorly understood. In particular, it is unclear whether disulfide bridging of TCRαβ proteins occurs before or after their association with CD3 chains (Bonifacino et al., 1988a; Manolios et al., 1991; Alarcon et al., 1988; Koning et al., 1988). The transmembrane domains of both TCRα and TCRβ proteins contain positively charged amino acid residues that would be expected to interfere with their assembly so that direct pairing of αβ polypeptides has been considered to be highly unfavorable (Green, 1991; Klausner et al., 1990). Recently, it was suggested that charge repulsions between TCRα and TCRβ transmembrane regions might be neutralized by assembly of individual α and β proteins with CD3 components, since the transmembrane region of each CD3 chain contains a negatively charged amino acid residue (Green, 1991; Cosson et al., 1991; Manolios et al., 1991). Evidence that individual TCRαβ polypeptides can interact directly with CD3 chains has been obtained from experiments in which fibroblasts were transfected with individual TCR cDNAs (Bonifacino et al., 1990; Cosson et al., 1991) and from studies of human T leukemias (Alarcon et al., 1988; Koning et al., 1988). However, direct evidence has been lacking that formation of disulfide-linked αβ dimers occurs after the association of individual TCRαβ proteins with CD3 components. Indeed, despite theoretical charge considerations, experiments using fibroblasts transfected with cDNAs encoding murine TCRα and TCRβ polypeptides demonstrated clearly that formation of disulfide-linked αβ dimers can occur efficiently in the absence of any other TCR components (Bonifacino et al., 1988a; Manolios et al., 1991).

Recently, we have found that formation of disulfide-linked αβ dimers is markedly limited in immature CD4⁺CD8⁺ thymocytes because nascent TCRα proteins are uniquely unstable in the ER of immature thymocytes, having a half-life of only 15 min in immature thymocytes, compared with a half-life in excess of 75 min in mature T cells (Kearse et al., 1994a). To enhance our understanding of the TCR assembly steps that are impaired in immature thymocytes, we undertook the present study to identify the molecular interactions that lead to formation of disulfide-linked αβ dimers in normal murine thymocytes and mature T cells.

Results

Two models can be considered regarding formation of αβ dimers in T cells: a CD3-independent αβ dimerization model and a CD3-associated αβ dimerization model (Figure 1). The two models are distinguishable in that the CD3-associated αβ dimerization model predicts that TCRα monomers are associated with CD3 components prior to their association with TCRβ chains (Figure 1, asterisk), and both TCRα and TCRβ monomers are assembled into a complex with CD3 components prior to αβ disulfide bonding (Figure 1, double asterisk). In the current study, we evaluate these two αβ dimerization pathways in immature and mature T cells.

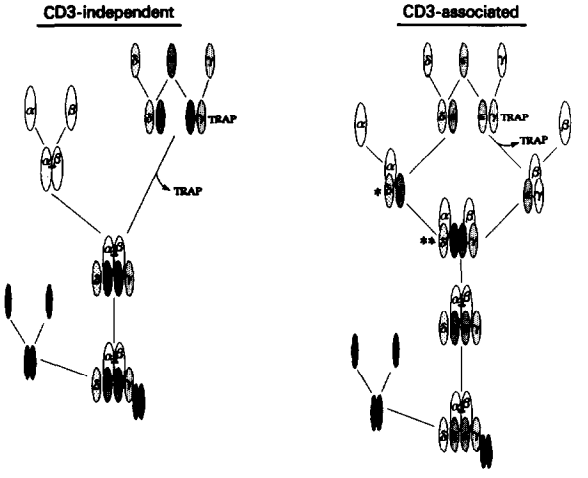

Figure 1. CD3-Independent and CD3-Associated αβ Dimerization Models

Model illustrating two pathways of disulfide-linked αβ dimer formation in murine T lymphocytes. In the CD3-independent model (left), α and β proteins disulfide link prior to assembly with CD3 components; consequently, nascent TCRα proteins assembled with CD3 chains exist exclusively as disulfide-linked αβ dimers. In contrast, the CD3-associated model predicts the existence of TCRα monomers that are associated with CD3 components prior to their association with TCRβ chains (right, asterisk), and also predicts that TCRα monomers associate with TCRβ chains prior to the formation of αβ disulfide bonds (right, double asterisk). Disulfide bonds are indicated by solid black bars.

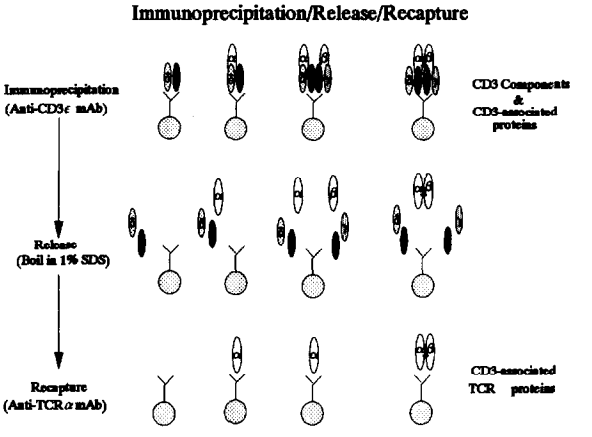

Figure 2. Immunoprecipitation/Release/Recapture Protocol

To assess the dimerization status of newly synthesized TCRα proteins associated with CD3 components, metabolically labeled lysates are first immunoprecipitated with anti-CD3ε MAb to capture CD3-associated TCRα proteins (immunoprecipitation); precipitates are then boiled in 1% SDS to release antibody-bound proteins (release); NP-40 lysis buffer is added to counteract the SDS detergent, and released proteins are then reprecipitated with anti-TCRα MAb to recapture TCRα molecules specifically (recapture). Analysis of recaptured material on SDS–PAGE gels under nonreducing conditions identifies the dimerization status of TCRα proteins originally assembled with CD3 components.

Monomeric TCRα Proteins Initially Assemble with CD3 Components in both Immature CD4$^+$CD8$^+$ Thymocytes and Splenic T Cells

Because formation of αβ dimers in both CD4$^+$CD8$^+$ thymocytes and splenic T cells involves the pairing of newly synthesized α partner chains with preexistent β partner chains (Kearse et al., 1994b), our initial studies focused on the assembly of newly synthesized α partner chains into disulfide-linked αβ dimers. To assess the dimerization status of CD3-associated TCRα proteins, we devised a novel immunoprecipitation/release/recapture protocol (Figure 2). In these experiments, metabolically labeled digitonin lysates were first immunoprecipitated with anti-CD3ε monoclonal antibody (MAb) to capture CD3-associated TCRα proteins; precipitates were then boiled in 1% SDS to release antibody-bound proteins; NP-40 lysis buffer was added to counteract the SDS detergent, and released proteins were then reprecipitated with anti-TCRα MAb to recapture TCRα molecules specifically (Figure 2). Analysis of recaptured material on SDS–PAGE gels under nonreducing conditions identifies the dimerization status of TCRα proteins originally assembled with CD3 components. Results of such immunoprecipitation/release/recapture experiments are presented in Figure 3. As is evident, metabolically labeled CD3-associated TCRα proteins did exist both as disulfide-linked αβ dimers and α monomers in CD4$^+$CD8$^+$ thymocytes and splenic T cells (Figure 3A), as predicted by the CD3-associated αβ dimerization model (Figure 1, asterisk). During the chase period, CD3-associated TCRα monomers became disulfide-linked to TCRβ proteins as the amount of CD3-associated α monomer decreased, while the amount of CD3-associated αβ dimer compensatorily increased (Figure 3A). The virtual absence of CD3-associated TCRα monomers in the chase samples serves to document that our experimental protocol, which does not expose immunoprecipitates to reducing agents such as 2-mercaptoethanol or dithiothreitol, preserves the integrity of disulfide-linked αβ dimers. Thus, these experiments demonstrate that nascent TCRα monomers assemble with CD3 proteins in both CD4$^+$CD8$^+$ thymocytes and mature T cells and, importantly, that CD3-associated TCRα monomers represent an intermediary in the formation of CD3-associated αβ heterodimers in both immature and mature T cells, two findings that are unique predictions of the CD3-associated αβ dimerization model (see Figure 1).

To confirm these results, we wished to assess the dimerization status of TCRα proteins at an early timepoint prior to their association with CD3. In this experiment, splenic T cells were metabolically labeled for only 5 min and TCRα proteins examined at various timepoints afterward by our immunoprecipitation/release/recapture protocol (Figure 3B). As expected, the vast majority of TCRα proteins at the end of the short pulse period were not CD3-associated, as they were not precipitated by anti-CD3ε MAb (Figure 3B). Interestingly, "free" TCRα proteins existed only as α monomers, even after sufficient time had elapsed for αβ dimers to form among CD3-associated TCRα proteins (Figure 3B, right lanes), a result consistent with the CD3-associated dimerization model (see Figure 1). Surprisingly, the 5 min metabolic labeling period was itself sufficient for a small number of TCRα proteins to become CD3-associated and even to form CD3-associated αβ dimers (Figure 3B, left panels). CD3-associated TCRα monomers and CD3-associated αβ dimers quantitatively

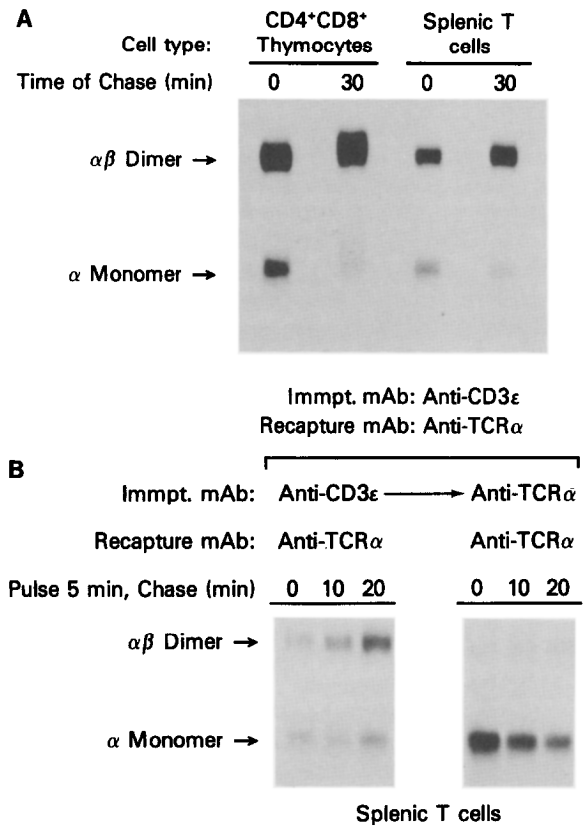

Figure 3. Both αβ Dimers and α Monomers Are Assembled with CD3 Chains in CD4+CD8+ Thymocytes and Splenic T Cells

(A) CD3-associated TCRα proteins were isolated from radiolabeled lysates of CD4+CD8+ thymocytes and splenic T cells as described in Figure 2. Samples were analyzed on one-dimensional SDS–PAGE gels under nonreducing conditions. The positions of TCRα monomers and TCRαβ dimers are indicated. Analysis of such precipitates on two-dimensional NEPHGE/SDS–PAGE gels revealed only radiolabeled TCRα proteins but not radiolabeled TCRβ proteins (Kearse et al., 1994b), indicating that each band solely reflected the amount of nascent TCRα proteins present. It should also be appreciated that the relative amounts of αβ dimerization occurring in splenic T cells relative to CD4+CD8+ thymocytes is markedly underestimated in these experiments because of unlabeled α protein pools, which exist in mature splenic T cells but do not exist in immature CD4+CD8+ thymocytes (Kearse et al., 1994b).

(B) Splenic T cells were pulse-labeled for 5 min and chased for the time period indicated. CD3-associated TCRα proteins and unassembled TCRα proteins were isolated from digitonin lysates and analyzed on one-dimensional SDS–PAGE gels under nonreducing conditions. The positions of TCRα monomers and TCRαβ dimers are indicated.

increased during the chase periods in amounts commensurate with the decrease in free TCRα monomers (Figure 3B), demonstrating the assembly of free TCRα monomers into CD3-associated TCRα monomers and then CD3-associated αβ dimers.

Monomeric TCRα Proteins Are Assembled with TCRβ Chains in both Immature CD4+CD8+ Thymocytes and Mature T Cells

The CD3-associated αβ dimerization model predicts the existence of two unique TCRα containing complexes not found in the CD3-independent αβ dimerization model: an

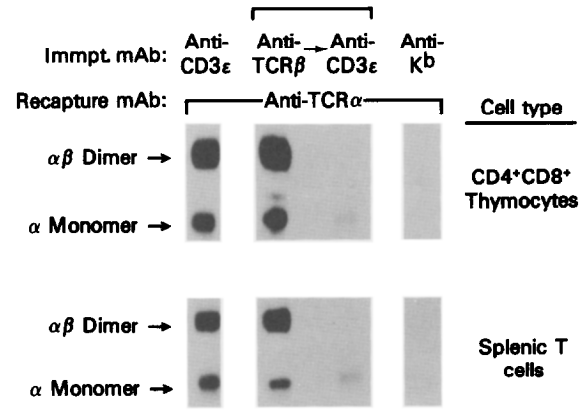

Figure 4. Monomeric TCRα Proteins Are Assembled with CD3 and TCRβ Chains in CD4+CD8+ Thymocytes and Splenic T Cells

Digitonin lysates of metabolically labeled CD4+CD8+ thymocytes and splenic T cells were precipitated with the indicated antibodies. Bound material was released by boiling in 1% SDS. Excess 1% NP-40 lysis buffer was added and released material was immunoprecipitated with anti-TCRα MAb to recapture TCRα proteins. Samples were analyzed on one-dimensional SDS–PAGE gels under nonreducing conditions. The positions of TCRα monomers and TCRαβ dimers are indicated.

intermediate complex containing TCRα monomers associated with CD3δε proteins (see Figure 1, asterisk), and a protein complex consisting of CD3-associated α and β monomers prior to the formation of the αβ disulfide bond (see Figure 1, double asterisk). Consequently, we next determined whether such complexes could be detected in immature CD4+CD8+ thymocytes and mature splenic T cells. In the experiment displayed in Figure 4, metabolically labeled TCRα proteins that had been released from either anti-CD3ε or anti-TCRβ immunoprecipitates were recaptured with anti-TCRα MAb and analyzed by one-dimensional SDS–PAGE under nonreducing conditions. As demonstrated, TCRα proteins were recaptured from both anti-CD3ε and anti-TCRβ immunoprecipitations, which existed as both disulfide-linked αβ dimers and as α monomers (Figure 4, lanes 1,2). In contrast, TCRα proteins were not captured from proteins released from anti-Kb immunoprecipitates, demonstrating the specificity of our release and recapture protocol (Figure 4, lane 4). Initial immunoprecipitation with anti-TCRβ MAb precleared CD3-associated αβ dimers isolated in sequential anti-CD3ε precipitations, as expected, since these complexes contained TCRβ chains (Figure 4, compare lanes 1 and 3). Most importantly, initial immunoprecipitation with anti-TCRβ MAb also precleared most CD3-associated α monomers from sequential anti-CD3ε precipitations, demonstrating that most CD3-associated α monomer complexes also contained β monomers prior to disulfide-bond formation, as predicted by the CD3-associated αβ dimerization model (see Figure 1, double asterisk). Furthermore, the small amount of CD3-associated α proteins that were not precleared by initial immunoprecipitation with anti-TCRβ MAb were captured in sequential anti-CD3ε precipitations and existed exclusively as α monomers (Figure 4, lane 3), as predicted (see Figure 1, asterisk). That these protein com-

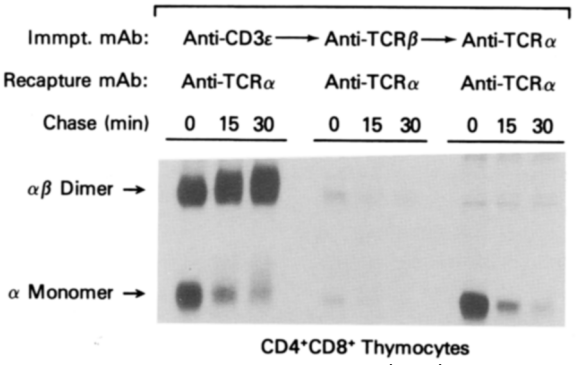

Figure 5. Two Pathways of αβ Dimerization

Digitonin lysates of metabolically labeled CD4+CD8+ thymocytes were sequentially immunoprecipitated with anti-CD3ε MAb, followed by anti-TCRβ MAb, followed by anti-TCRα MAb. That immunoprecipitations by each specific MAb went to completion was verified by analysis on SDS–PAGE gels (data not shown). Immunoprecipitated material was released by boiling in SDS and recaptured with anti-TCRα MAb. Samples were analyzed on one-dimensional gels under nonreducing conditions. The positions of TCRα monomers and TCRαβ dimers are indicated.

plexes consisted of TCRα monomers associated with CD3δε proteins was verified by precipitation with anti-CD3δ-specific antibody (see Figures 6A and 6B).

Two Pathways of αβ Dimer Formation

To examine simultaneously all possible pathways of αβ dimer formation in immature CD4+CD8+ thymocytes, we performed an immunoprecipitation/release/recapture experiment on lysates from metabolically labeled CD4+CD8+ thymocytes in which lysates were sequentially immunoprecipitated with anti-CD3ε MAb to capture CD3-associated TCRα proteins, followed by anti-TCRβ MAb to capture CD3-independent TCRα proteins, and finally with anti-TCRα MAb, to capture free TCRα proteins. Immunoprecipitated material was released by boiling in SDS and then reprecipitated with anti-TCRα MAb to recapture nascent TCRα chains. Significant amounts of nascent disulfide-linked αβ dimers and α monomers were associated with CD3 components in CD4+CD8+ thymocytes (Figure 5, left lanes). However, some number of αβ dimers and α monomers were also recaptured from sequential anti-TCRβ precipitates of CD4+CD8+ thymocyte lysates (Figure 5, middle lanes), indicating that some α monomers associate with TCRβ proteins and are assembled into αβ dimers independently of their association with CD3 components. As expected, free TCRα proteins captured in sequential anti-TCRα precipitates existed exclusively as α monomers (Figure 5, right lanes). Note that the faint upper bands present in the final anti-TCRα precipitates were not αβ dimers, as the protein bands were insensitive to endoglycosidases (data not shown). The number of CD3-associated TCRαβ dimers increased during the chase period, and this increase could be quantitatively accounted for by decreased numbers of CD3-associated α monomers (Figure 5, left lanes), but could not be quantitatively accounted for by decreased numbers of CD3-independent αβ dimers (Figure 5, middle lanes). Thus, these data indicate that the CD3-associated pathway is the predominant pathway of αβ dimer formation in CD4+CD8+ thymocytes, although a small amount of CD3-independent αβ dimerization occurs. Similar results were obtained in splenic T cells (data not shown).

It might also be noted that, unlike splenic T cells in which most free TCRα monomer proteins were stable (see Figure 3B), most free TCRα monomer proteins that failed to associate with either CD3 or TCRβ chains were degraded in immature CD4+CD8+ thymocytes within 15–30 min, as their loss during the chase period was not accompanied by a commensurate increase in TCRα monomer proteins associated with either CD3 or TCRβ chains (Figure 5, compare right lanes with left and middle lanes). These results confirm our previous findings regarding the unique instability of nascent TCRα proteins within the ER of immature CD4+CD8+ thymocytes (Kearse et al., 1994b).

Formation of αδε and βγε Protein Complexes in CD4+CD8+ Thymocytes

In our next set of studies, we evaluated the formation of protein complexes consisting of nascent αδε and βγε polypeptides, as predicted by the CD3-associated dimerization model (see Figure 1). In these studies, we utilized anti-CD3δ and anti-CD3γε antibodies, but we could not utilize our immunoprecipitation/release/recapture protocol because our anti-TCRβ MAb could not recapture denatured TCRβ chains. Digitonin lysates of metabolically labeled CD4+CD8+ thymocytes were immunoprecipitated with anti-CD3ε, anti-CD3δ, or anti-CD3γε antibodies and subjected to one-dimensional SDS–PAGE analysis under reducing conditions (Figure 6A). TCRα and TCRβ proteins in anti-CD3ε precipitates could be distinguished from one another because of differential processing of their N-linked oligosaccharide sidechains (Kearse and Singer, 1994), even though their core protein sizes after endoglycosidase H (Endo H) digestions were indistinguishable (Figure 6A, left lanes). More importantly, it can be seen that nascent TCRα proteins were precipitated by anti-CD3δ antibody but nascent TCRβ proteins were not; rather, nascent TCRβ proteins were isolated in subsequent precipitations with anti-CD3γε MAb (Figure 6A, right lanes). These results were confirmed by analysis of such precipitates on two-dimensional NEPHGE/SDS–PAGE gels, which separate TCRα and TCRβ proteins based on both charge and molecular weight differences (Figure 6B). Similar results were obtained in splenic T cells (data not shown).

Thus, these data demonstrate that all nascent CD3-associated TCRα proteins were assembled into complexes containing CD3δ chains, including αδε and αβγδε complexes, and indicate the existence of βγε complexes, as predicted by the CD3-associated dimerization model (see Figure 1).

TRAP Association with CD3γε but Not CD3δε Pairs

TRAP is thought to be a molecular chaperone and to function in intracellular transport or assembly of the TCR complex. Consequently, we examined the association of TRAP with CD3 components. Digitonin lysates of metabolically

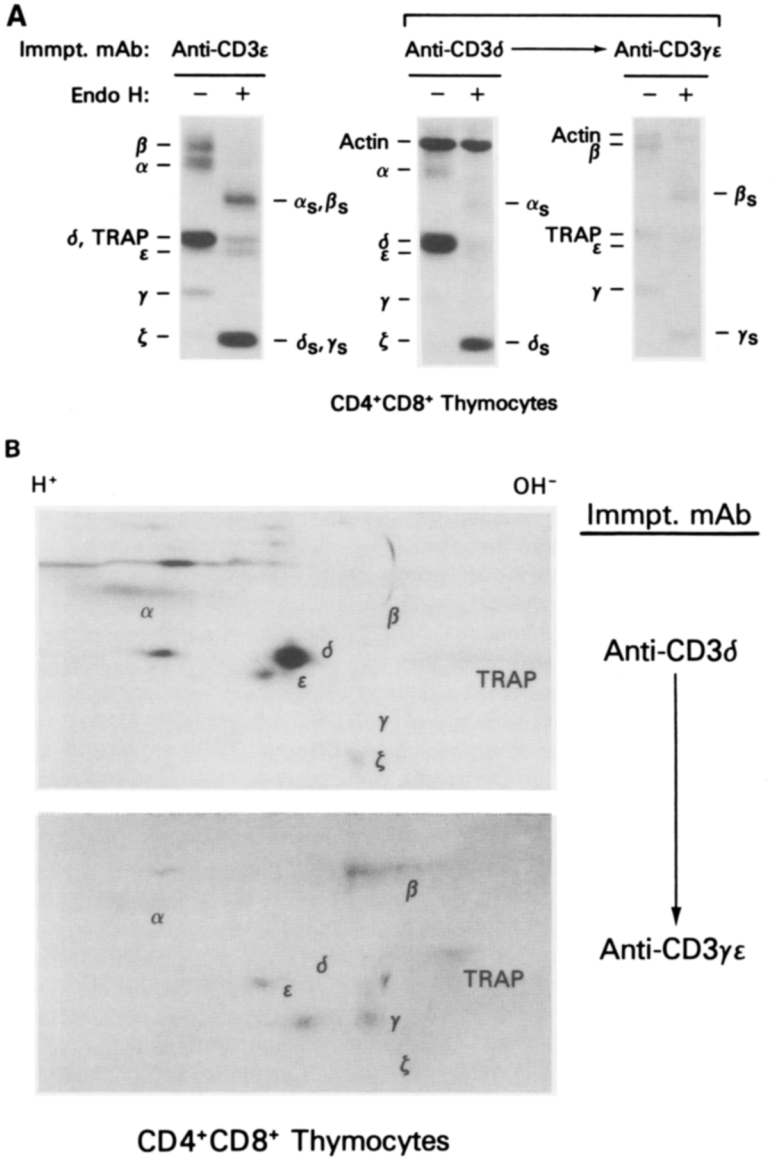

Figure 6. Formation of βγε Complexes in Immature CD4+CD8+ Thymocytes

(A) Digitonin lysates of metabolically labeled CD4+CD8+ thymocytes were immunoprecipitated with anti-CD3ε MAb (left lanes), anti-CD3γε MAb (middle lanes), and anti-CD3δ antisera, followed by anti-CD3γε-specific MAb (right lanes). Precipitates were digested with Endo H and analyzed under reducing conditions. The positions of TCR proteins are indicated. Note that nascent TCRα proteins, but not nascent TCRβ proteins, are isolated by anti-CD3δ antibody.

(B) Digitonin lysates of metabolically labeled CD4+CD8+ thymocytes were sequentially immunoprecipitated with anti-CD3δ antibody (top), followed by precipitation with anti-CD3γε MAb (bottom). Precipitates were analyzed on two-dimensional NEPHGE/SDS–PAGE gels under reducing conditions. The positions of TCR proteins are indicated.

labeled CD4+CD8+ thymocytes were precipitated with anti-CD3ε, anti-CD3δ, and anti-CD3γε antibodies and resolved by one-dimensional SDS–PAGE under nonreducing conditions. Although CD3δ, CD3ε, and TRAP proteins are of similar molecular weight, analysis under nonreducing conditions separates TRAP from CD3δ and CD3ε proteins, because these latter proteins exhibit increased mobility under nonreducing conditions because of intrachain disulfide bonds (Bonifacino et al., 1988b). As is evident, TRAP was immunoprecipitated by anti-CD3γε MAb but was not precipitated by anti-CD3δ antibody (Figure 7). Identical results were obtained in both CD4+CD8+ thymocytes and splenic T cells. Thus, TRAP associates with CD3γε proteins but not with CD3δε proteins. As precipitation with anti-TCRβ MAb never precipitates TRAP from either CD4+CD8+ thymocytes or splenic T cells (data not shown), we conclude that TRAP dissociates from CD3γε complexes prior to their assembly with TCRβ proteins.

Decreased Association of TCRα Proteins with CD3 Components in CD4+CD8+ Thymocytes Compared with Splenic T Cells

Because CD3-assembled TCRα monomers represent an important intermediary in αβ dimer formation, we wished to examine quantitatively the association of newly synthesized TCRα proteins with CD3 components in CD4+CD8+ thymocytes relative to splenic T cells. For these studies, metabolically labeled cell lysates from CD4+CD8+ thymocytes and splenic T cells were immunoprecipitated with anti-CD3ε MAb and analyzed on two-dimensional NEPHGE/SDS–PAGE gels under reducing conditions. It can be seen that fewer TCRα proteins associated with CD3 components in CD4+CD8+ thymocytes than in splenic T cells (Figure 8, top). Indeed, even though assembly of TCRα proteins is quantitatively underestimated in mature splenic T cells because of competition from preexisting pools of unlabeled α proteins (Kearse et al., 1994b), the number

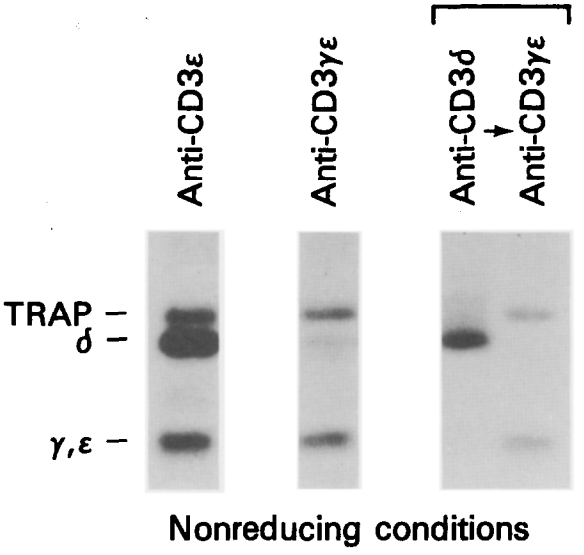

Figure 7. TRAP Associates Specifically with CD3γε Pairs but Not CD3δε Pairs in CD4⁺CD8⁺ Thymocytes

Digitonin lysates of metabolically labeled CD4⁺CD8⁺ thymocytes were immunoprecipitated with anti-CD3ε MAb (left lanes), anti-CD3γε MAb (middle lanes), or anti-CD3δ antibody, followed by anti-CD3γε MAb (right lanes). Immunoprecipitates were analyzed on one-dimensional SDS–PAGE gels under nonreducing conditions. The positions of TCR proteins are indicated.

of radiolabeled TCRα proteins that associated with CD3 components in immature CD4⁺CD8⁺ thymocytes during the metabolic labeling period was still only 20% of that in mature splenic T cells. Thus, the initial assembly step in αβ dimer formation, namely association of newly synthesized TCRα proteins with CD3 components, is significantly diminished in immature CD4⁺CD8⁺ thymocytes.

To determine whether TCRα proteins associate with CD3δ proteins independently of CD3ε chains in immature and mature T cells, an association that has been demonstrated in fibroblasts transfected with α and δ cDNAs (Bonifacino et al., 1990; Cosson et al., 1991; Manolios et al., 1991), material not precipitated by anti-CD3ε MAb was sequentially immunoprecipitated with anti-CD3δ antibody (Figure 8). As is evident, all nascent TCRα proteins were assembled with CD3δε pairs in both CD4⁺CD8⁺ thymocytes and splenic T cells, as no nascent TCRα proteins were visible in sequential anti-CD3δ precipitates of either cell type (Figure 8, bottom). Interestingly, unlike mature T cells, most CD3δ chains in immature CD4⁺CD8⁺ thymocytes existed as CD3ε-associated proteins with only a minority existing as free CD3δ chains (Figure 8). The significance of this difference between immature and mature T cells is uncertain, but it may reflect the possibility that pairing of CD3δ proteins with CD3ε chains is enhanced under conditions in which TCRα proteins are limiting.

Stabilization of Nascent TCRα Proteins in Immature Thymocytes by Association with CD3 Components

The assembly of nascent TCRα monomers with CD3 components, and their subsequent disulfide linkage with TCRβ proteins in CD4⁺CD8⁺ thymocytes (see Figure 3A), indicated that association of TCRα monomers with CD3δε chains stabilized nascent TCRα proteins in immature thymocytes. To verify this issue, metabolically labeled lysates of immature CD4⁺CD8⁺ thymocytes were sequentially immunoprecipitated with anti-CD3ε MAb (to isolate CD3-assembled α proteins), followed by immunoprecipitation with a mixture of anti-TCRα and anti-TCRβ MAbs (to isolate all unassembled TCRα and TCRβ proteins) (Figure 9, top and middle). Immunoprecipitates were analyzed on two-dimensional NEPHGE/SDS–PAGE gels under reducing conditions. To aid in visualization and quantitation of TCRα proteins, size and charge microheterogeneity among TCRα proteins were minimized by treatment of immunoprecipitates with PNGase F glycosidase to remove oligosaccharide side chains. As is evident, most nascent TCRα proteins synthesized in immature CD4⁺CD8⁺ thymocytes did not survive 30 min of chase, whereas most TCRβ and CD3ε proteins did survive (Figure 9, bottom). The instability of nascent TCRα proteins in immature thymocytes was related to its assembly status, as unassembled TCRα proteins were rapidly degraded during the chase period (Figure 9, middle) but CD3-associated α proteins were clearly stable (Figure 9, top). Thus, we conclude that formation of CD3-associated α complexes stabilizes nascent TCRα proteins in immature CD4⁺CD8⁺ thymocytes.

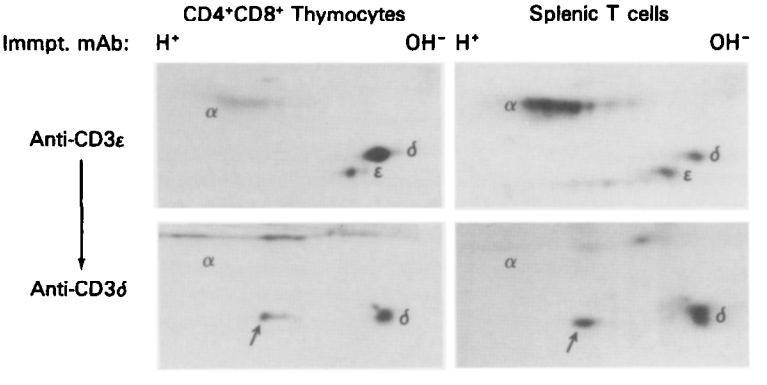

Figure 8. Nascent TCRα Proteins Assemble with CD3δε Pairs in CD4⁺CD8⁺ Thymocytes and Splenic T Cells

Digitonin lysates of metabolically labeled CD4⁺CD8⁺ thymocytes and splenic T cells were sequentially immunoprecipitated with anti-CD3ε MAb, followed by anti-CD3δ antibody. Precipitates were analyzed on two-dimensional NEPHGE/SDS–PAGE gels under reducing conditions. The positions of CD3δ, CD3ε, and TCRα proteins are indicated. Arrow indicates an unknown protein, which specifically coprecipitates with free CD3δ proteins in both immature and mature T cells.

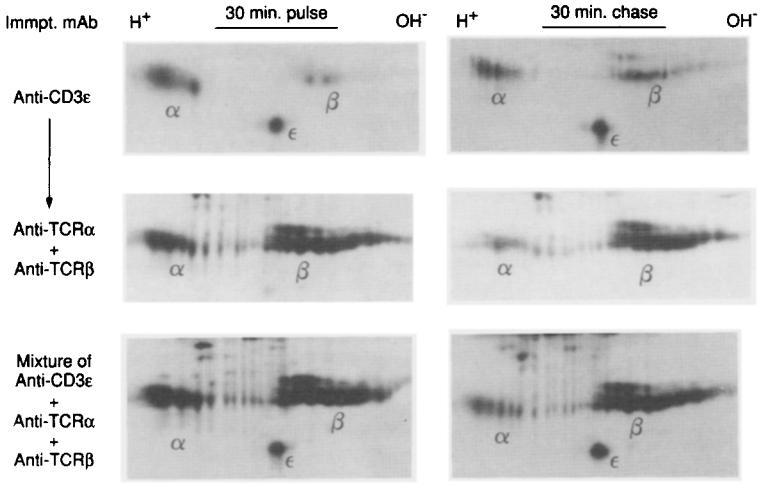

Figure 9. Assembly of Nascent TCRα Proteins with CD3 Chains Stabilizes TCRα Proteins in Immature CD4⁺CD8⁺ Thymocytes

Digitonin lysates of metabolically labeled CD4⁺CD8⁺ thymocytes were sequentially immunoprecipitated with anti-CD3ε MAb to capture TCRα proteins assembled with CD3, followed by immunoprecipitation with both anti-TCRα plus anti-TCRβ MAb to capture unassembled TCRα and TCRβ proteins. Precipitates were treated with PNGase F to remove N-linked oligosaccharides and analyzed on two-dimensional NEPHGE/SDS–PAGE gels under reducing conditions. The positions of TCR proteins are indicated. It should be noted that the radiolabeled β chains associated with CD3 components in these experiments are not assembled into disulfide-linked αβ dimers, but represent partial βγε complexes not assembled with TCRα proteins in immature CD4⁺CD8⁺ thymocytes (data not shown).

Discussion

The current study has examined and compared the molecular interactions involved in formation of disulfide-linked αβ heterodimers in immature CD4⁺CD8⁺ thymocytes and mature splenic T cells. Rather than occurring spontaneously and independently of other protein interactions, we found that formation of most disulfide-linked αβ dimers occurred only after association of nascent TCRα monomer proteins with CD3δε components and subsequent association with TCRβ–γε trimers. Association of TCRα monomer proteins with CD3δε components was quantitatively diminished in immature CD4⁺CD8⁺ thymocytes relative to mature splenic T cells. Interestingly, we found that association of TCRα monomers with CD3 components stabilized nascent TCRα proteins, which otherwise were rapidly degraded within the ER of immature CD4⁺CD8⁺ thymocytes. Thus, the present study provides direct evidence that association of TCRα monomer proteins with CD3δε components is the initial step in αβ dimer formation in normal murine T cells and demonstrates that association of nascent TCRα proteins with CD3δε chains is a limiting step in αβ dimer formation in immature CD4⁺CD8⁺ thymocytes.

The role of CD3 components in disulfide bond formation between TCRα and TCRβ polypeptides has been controversial, as evidence exists for both CD3-independent and CD3-associated αβ dimerization pathways in various cell types, including T hybridoma variants, T cell leukemias, and nonlymphoid cells transfected with cDNAs encoding TCR proteins (Bonifacino et al., 1988a; Manolios et al., 1991; Alarcon et al., 1988; Koning et al., 1988). As shown in the present study, determination of the molecular interactions involved in αβ dimerization in normal T cells is complicated by the potential existence of alternative assembly pathways and by differential stabilities of unassembled α and β proteins in the ER of immature thymocytes and mature T cells. To overcome these problems, we devised a novel immunoprecipitation/release/recapture protocol, which allowed us to identify rigorously the disulfide-linkage status of unassembled and assembled TCRα proteins. Indeed, we detected in both immature thymocytes and mature T cells two important assembly intermediates containing TCRα proteins that exist prior to the formation of αβ disulfide bonds and consist of CD3-associated α monomers, and CD3-associated α monomers further complexed to β monomer proteins. These results are consistent with previous observations that non-disulfide-linked TCRα proteins can associate with CD3 components (Alarcon et al., 1988; Koning et al., 1988; Manolios et al., 1991), but the present results importantly extend these observations by demonstrating that CD3-associated TCRα monomer proteins are, in fact, intermediates in the αβ dimerization pathway and are present in normal T cells. Consistent with the CD3-associated pathway of αβ dimerization, we also detected protein complexes consisting of nascent TCRβ proteins associated with CD3γε chains, which existed independently of nascent TCRα proteins. Because of significant amounts of preexistent TCRβ protein pools in both CD4⁺CD8⁺ thymocytes and splenic T cells (Kearse et al., 1994b), we were unable to demonstrate in the present study that such βγε complexes represented true intermediates in the αβ dimerization pathway. However, we believe that such βγε complexes do, in fact, represent intermediates in the TCR assembly pathway, since mature TCR complexes contain these proteins.

From transfection studies of chain pairing (Cosson et al., 1991; Manolios et al., 1991), it is known that TCRα monomer proteins are able to associate directly with CD3δ chains. Consequently, initial association of α monomers with CD3 components in immature thymocytes and mature T cells may involve initial association of TCRα and CD3δ chains, which then rapidly complex with CD3ε chains to form αδε complexes. This point remains speculative, since we were unable to detect formation of CD3ε-independent dimers of α and δ in either immature thymocytes or mature splenic T cells. In fact, we noted during the course of these studies that the majority of nascent

CD3δ proteins exist in association with CD3ε chains in immature CD4+CD8+ thymocytes, but exist as free CD3δ proteins in mature splenic T cells. The significance of this finding remains to be determined, but we think that formation of CD3δε dimers may be a secondary consequence of the diminished availability of TCRα proteins in immature CD4+CD8+ thymocytes, since we have observed formation of similar δε complexes in mature T cells under conditions in which nascent TCRα proteins are destabilized and rapidly degraded (unpublished data).

The present study identifies the initial association of TCRα monomer proteins with CD3 components as an unexpected and limiting step in the formation of disulfide-linked αβ dimers in immature CD4+CD8+ thymocytes. That the initial association of TCRα monomer proteins with CD3 components is limiting in immature CD4+CD8+ thymocytes provides an explanation for our previous observation that αβ dimer formation in immature CD4+CD8+ thymocytes is as profoundly decreased for a matched pair of TCRα and TCRβ transgenic proteins as for random pairs of TCRα and TCRβ endogenous proteins (Kearse et al., 1994b). If the fate of newly synthesized TCRα proteins in the ER of immature CD4+CD8+ thymocytes were the outcome of a race between αβ dimerization and ER degradation, matched pairs of α and β transgenic proteins should have been much more successful in avoiding ER degradation than random pairs of α and β endogenous proteins. However, the present study demonstrates that survival of newly synthesized TCRα proteins in the ER of immature CD4+CD8+ thymocytes depends upon their initial association with CD3 components prior to their dimerization of β proteins, which would not be affected by whether the α and β proteins were matched or randomly paired.

Finally, the present study documents that TRAP, believed to function in intracellular transport or assembly of the TCR complex, selectively associates with CD3γε proteins but not CD3δε proteins in both immature and mature T cells. This demonstrates that TRAP specifically associates with CD3γε pairs in any murine T cell type and significantly limits the potential role that TRAP can perform in TCR assembly to formation of βγε intermediates. Interestingly, the human analogue of TRAP, the ω protein, has been reported to associate with numerous TCR components in human T cell lines, including TCRα proteins, TCRβ proteins, and CD3δε chains (Alarcon et al., 1988; Pettey et al., 1987). The reason for these differences are unknown, but may reflect different assembly requirements in human and murine T lymphocytes.

In conclusion, the present study documents that CD3 components play a direct role in the formation of most disulfide-linked αβ dimers in normal T cells and identifies the initial association of TCRα monomer proteins with CD3 components as a limiting step in the formation of disulfide-linked αβ dimers in immature CD4+CD8+ thymocytes.

Experimental Procedures

Animals and Cell Preparation

C57BL/6 (B6) mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, Maine). CD4+CD8+ thymocytes were isolated by their adherence to plastic plates coated with anti-CD8 MAb (83-12-5), and were typically > 96% CD4+CD8+ as described (Bonifacino et al., 1990). Purified splenic T cells were obtained by incubating single cell suspensions of spleen cells on rabbit anti-mouse immunoglobulin (Organon Technika–Cappel, Malvern, Pennsylvania)-coated tissue culture plates for 60 min at 37°C, followed by isolation of nonadherent cells. The resultant cell populations were > 95% CD3ε+ as determined by surface staining with MAb to CD3ε.

Metabolic Labeling and Immunoprecipitation

For metabolic labeling, cells were resuspended at 10 × 10⁶ cell/ml in methionine-free RPMI 1640 media (Biofluids, Rockville, Maryland) containing 10% fetal calf serum and 10 mCi [^{35}S]methionine (trans ^{35}S label; ICN Biomedical, Irvine, California) for 30 min at 37°C. Immunoprecipitation was performed following solubilization of cells in lysis buffer (20 mM Tris, 150–300 mM NaCl, 10 mM iodoacetamide, 20 μg/ml leupeptin, 40 μg/ml aprotinin) containing 1% digitonin (Wako) or 1% NP-40 (Calbiochem) for 25 min on ice. Cell lysates were centrifuged to remove insoluble material and lysates mixed with appropriate antibodies preabsorbed to protein A–Sepharose (Pharmacia) or protein G–Sepharose (Pharmacia) and incubated for 3 hr at 4°C. Precipitates were washed three times in lysis buffer containing 0.2% detergent, followed by one wash in phosphate-buffered saline. Sequential precipitations and glycosidase treatment were performed as previously described (Kearse et al., 1993).

Immunoprecipitation/release/recapture experiments were performed as follows. Samples were solubilized in 1% digitonin lysis buffer and immunoprecipitated as described above. Precipitates were solubilized in 50 μl of 1% SDS, boiled for 5 min, and 1 ml of 1% NP-40 lysis buffer was added. Samples were cooled on ice for 5 min, then centrifuged; supernatants were removed and precleared for 60 min with protein A–Sepharose beads. Samples were centrifuged again, supernatants removed, and precipitated with antibody absorbed to protein A–Sepharose. Secondary precipitates were washed three times in 1% lysis buffer containing 0.2% NP-40, followed by one wash in phosphate-buffered saline. Recovery of material in such release/recapture experiments was > 90% of that isolated in primary immunoprecipitations, and analysis by two-dimensional NEPGHE/SDS–PAGE gels revealed exclusively radiolabeled TCRα protein but not radiolabeled TCRβ protein (data not shown). The following MAbs were used for immunoprecipitation in this study: anti-K^b, anti-CD3ε 145-2C11 (Leo et al., 1987), anti-CD3γε 7D6 (Coulie et al., 1991), anti-TCRα H28-710 (Becker et al., 1989), and anti-TCRβ H57-597 (Kubo et al., 1989). The following antiserum was used: anti-CD3δ R9 antiserum (Samelson et al., 1986).

Gel Electrophoresis and Immunoblotting

Samples were analyzed on two-dimensional nonreducing × reducing SDS–PAGE gels and two-dimensional NEPHGE/SDS–PAGE gels as previously described (Kearse et al., 1994a). For immunoblotting, samples were probed with antisera in phosphate-buffered saline containing 5% milk and 0.02% NaN_3, followed by ^{125}I-labeled protein A (10 μCi/ml) (ICN Biomedical, Irvine, California). Multiple autoradiographs of different exposures were scanned to ensure linearity of densitometry analysis.

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